

# Apoptosis of rat hepatic stellate cells induced by anti-focal adhesion kinase antibody

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## Abstract

**AIM:** To explore the role of focal adhesion kinase (FAK) in the apoptosis in culture-activated rat hepatic stellate cells (HSCs) using a specific anti-FAK antibody.

**METHODS:** Rat HSCs were prepared from Wistar rats by *in situ* perfusion of collagenase and pronase and single-step density Nycodenz gradient. Culture-activated HSCs were serum-starved and treated with the anti-FAK antibodies for 24, 48 or 72 h. The apoptosis of HSC was detected by DNA-fragment assay, flow cytometry and caspase-3 activity determination. The expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) mRNA was assessed by reverse transcription polymerase chain reaction (RT-PCR).

**RESULTS:** The experiment showed that anti-FAK antibodies induced apoptosis of culture-activated rat HSCs. This phenomenon displayed the classical features of apoptotic cell death (DNA fragmentation, cell cycle analysis) after treated with 30 mg·L<sup>-1</sup> FAK antibody for 72 h, and accompanied by a significant increase of caspase-3 activity (1208±76) vs (309±28) nmol·min<sup>-1</sup>·g<sup>-1</sup>, *t*=208.5, *P*<0.05. Meanwhile, treatment with the FAK antibody in HSCs could markedly decrease the TIMP-1 mRNA expression (0.07±0.01 vs 0.38±0.03, *t*=2.72, *P*<0.05).

**CONCLUSION:** FAK plays an important role in the survival of HSCs and the specific anti-FAK antibody could induce the apoptosis in rat HSCs.

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## INTRODUCTION

Focal adhesion kinase (FAK) is a non-receptor tyrosine ubiquitously expressed in cells. It was initially shown to be the initiator of focal adhesion formation in adherent cells, after its binding to integrins which induce its autophosphorylation<sup>[1]</sup>.

However, it can also be activated by a great variety of other stimuli being able to act on different intracellular signaling, and neuropeptides<sup>[2-4]</sup>. Its autophosphorylation is followed by a submembranous localization which is crucial for the biological roles of FAK, including cell spreading, migration, proliferation, survival and prevention of apoptosis<sup>[5-7]</sup>. Proteolytic cleavage of FAK by caspase-3 has been reported during growth factor deprivation-induced apoptosis in human umbilical vein endothelial cells<sup>[8]</sup>, which implies an association between FAK and apoptosis<sup>[9,10]</sup>. The pathologic basis of hepatic cirrhosis is fibrosis and hepatic stellate cells (HSC) are presently regarded as one of the key cell types involved in the progression of liver fibrosis<sup>[11-13]</sup>. The perpetuation of HSC activation leads to an increased number of collagen-producing cells and finally to the accumulation of extracellular matrix (ECM)<sup>[14-16]</sup>. Therefore, the strategy for terminating the proliferation of activated HSC by apoptosis might be an exciting therapy for patients with chronic liver injury and fibrosis<sup>[17-19]</sup>.

FAK has also been shown to play an important role in the HSC activation<sup>[20]</sup>. PLC  $\gamma$  recruitment by FAK during HSC adhesion is an important process implicating a link between integrin and PDGF-mediated signal pathways to regulate HSC adhesion and mobility<sup>[21]</sup>. An adherence dependent pp125FAK-paxillin signaling pathway in fibroblasts inhibited damage-induced apoptosis<sup>[22]</sup>. Thus, we hypothesized that the modulation of biological roles of FAK by a neutralizing anti-FAK antibody might stop the fibroproliferative response and induce apoptosis in HSC.

## MATERIALS AND METHODS

### Materials

Male Wistar rats were obtained from the Experimental Animal Center of West China Medical Center of Sichuan University (West China University of Medical Sciences, Chengdu, Sichuan). Dulbecco's modified medium (DMEM), Trypsin-EDTA and new born calf serum (CS) were from GibcoBRL (Maryland, USA). Pronase, Collagenase B and DNAase I were from Roche Molecular Biochemicals, (Mannheim, Germany). Nycodenz was from Sigma (ST. Louis, USA). Antibodies to Desmin,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) were obtained from Dako (Glostrup, Denmark). Affinity-purified polyclonal antibody to FAK (epitope mapping at the carboxy terminus of focal adhesion kinase) were purchased from Santa Cruz (Santa Cruz, USA). The caspase-3 cellular activity assay kit was purchased from CalBiochem-Novabiochem Corporation (San Diego, USA).

### Methods

**HSC isolation and apoptosis induction** HSCs were isolated from male Wistar rats by *in situ* pronase-collagenase perfusion and single-step Nycodenz gradient<sup>[23]</sup>. The cells were seeded at a density of 1.5×10<sup>5</sup>/cm<sup>2</sup> on glass coverslips in 6-well culture plate or 100-mm dishes (Falcon) and maintained in DMEM containing 200 ml·L<sup>-1</sup> heat-inactivated new-born calf serum. The purity of HSC preparations was assessed by

intrinsic vitamin A autofluorescence and immunocytochemistry with antibody against desmin. The viability of the cells was evaluated by the Trypan blue dye exclusion test. The purity and viability of the primary cells exceeded 90 % and 95 %, respectively. Therefore, HSC cultured on uncoated plastic dishes spontaneously acquired an activated phenotype, characterized by expression of  $\alpha$ -SMA and by loss of vitamin A droplets<sup>[24,25]</sup>. After reaching confluency (about 10-14 d after plating), activated HSC were detached by incubation with trypsin, and split in a 1:2 ratio. Experiments were performed on cells between the second and 5<sup>th</sup> passages using 3 independent cell lines, and the purity of activated HSC exceeded 98 %. HSC ( $5 \times 10^6$ ) were plated in uncoated plastic dishes for 4 h and the medium was changed to serum-free DMEM for 24 h to synchronize the HSC in the G<sub>1</sub> phase of the cell cycle<sup>[26]</sup>. The antibodies against FAK was filter-sterilized and added to the serum-free DMEM medium containing 1 g · L<sup>-1</sup> bovine serum albumin (the final concentration of anti-FAK antibodies was 30 mg · L<sup>-1</sup>). The analysis of apoptosis was carried out after 24-72 h of incubation with the antibodies. The serum-free DMEM medium containing the antibodies was changed every 24 h.

**Analysis of DNA fragmentation** HSC from the anti-FAK antibodies treated was pooled for DNA fragmentation analysis. A DNA fragmentation assay was performed as described previously<sup>[27]</sup>. In brief, HSC was gently lysed for 30 min at 48 °C in a buffer containing 5 mmol · L<sup>-1</sup> Tris buffer (pH7.4), 20 mmol · L<sup>-1</sup> EDTA, and 5 ml · L<sup>-1</sup> Triton X-100. After centrifugation at 15 000r · min<sup>-1</sup> for 15 min, supernatants containing soluble fragmented DNA were collected and treated with RNAase (20 mg · L<sup>-1</sup>), followed by proteinase K (20 mg · L<sup>-1</sup>) digestion. DNA fragments were precipitated in 990ml · L<sup>-1</sup> ethanol. Samples were then electrophoresed on a 20g · L<sup>-1</sup> agarose gel, visualized with 1 g · L<sup>-1</sup> ethidium bromide and photographed under short-wave ultraviolet light.

**Flow cytometry** Cell viability was determined using trypan blue dye exclusion, and the existence of apoptotic cells was confirmed as well by the appearance of a sub-G<sub>0</sub>/G<sub>1</sub> peak fraction in the cell cycle analysis<sup>[28]</sup>. For the cell cycle analysis, ethanol-fixed cells were stained with propidium iodide (50 mg · L<sup>-1</sup>) in the presence of RNase A (100 mg · L<sup>-1</sup>), and then analyzed using the fluorescence-activated cell sorter (FACS, Coulter, EPICS ELITE ESP model), with a cell cycle analysis program.

**Cellular caspase-3 activity determination** The cellular caspase-3 activity assay from Calbiochem-Novabiochem Corporation measures the colorimetric reaction of the cleavage of the amino acid motif DEVD, thereby releasing the chromophorep-nitroanilide<sup>[29]</sup>. Following phosphate-buffered saline washing, cell lysate was prepared according to the manufactures' instructions. The level of caspase-3 enzymatic activity on the cell lysate is directly proportional to the color reaction that was quantitated spectrophotometrically at a wavelength of 405 nm, using a microplate reader for 96 wells (Bio Rad, model 550). And the total protein content of each cell lysate was determined by the Coomassie dye binding assay (Bradford method). Data were corrected for background (no substrate or no cell lysate) and caspase-3 activities were expressed as nmol · min<sup>-1</sup> · g<sup>-1</sup> of protein.

**TIMP-1 mRNA detection by RT-PCR** The total RNA was isolated from HSC using Trizol reagent (Life Technologies, Inc, USA), precipitated in ethanol and resuspended in sterile RNAase-free water for storage at -70 °C, as described previously<sup>[30]</sup>. One-step reverse transcription-polymerase chain reaction (RT-PCR) was performed according to the method of the supplier (Titan<sup>TM</sup> one tube RT-PCR kit, Roche Molecular

Biochemicals). Primers for rat tissue inhibitors metalloproteinase-1 (TIMP-1) and GAPDH were designed using the Primer3 program from Whitehead Institute for Biomedical Research (Cambridge, MA, USA)<sup>[31]</sup>, synthesized and purified by HPLC in Gibco BRL Custom Primers (HongKong). Primer sequences were as follows: TIMP-1 sense, 5'-GAC CTG GTC ATA AGG GCT AAA-3'; antisense, 5'-GCC CGT GAT GAG AAA CTC TTC ACT-3'; GAPDH sense, 5'-ACC ACA GTC CAT GCC ATC AC-3'; antisense, 5'-TCC ACC ACC CTG TTG CTG TA-3', and the expected size was 216 bp and 452 bp respectively. One microgram RNA was added to each reaction and the RT-PCR was performed in the following steps: reverse-transcription was performed at 50 °C for 30 min; and amplification was performed in a thermal controller (model PTC-100, MJ Research, Watertown, USA) for 35cycles (denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 1 min), and 10 min at 72 °C for final extension after the last cycle. 5  $\mu$ l of the PCR products was analyzed by 20 g · L<sup>-1</sup> agarose gel electrophoresis with TAE buffer at 80V for 40 min, visualized with ethidium bromide and photographed under UV light. The semi-quantitative analysis was performed. TIMP-1/GAPDH quotient is the indication of TIMP-1. Experiments were performed at least three times with similar results.

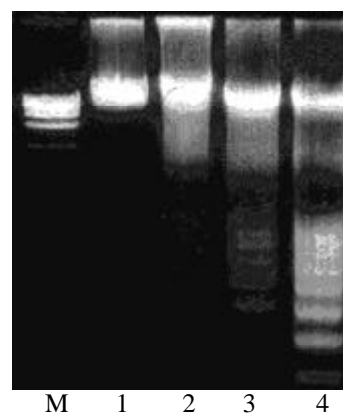
### Statistical analysis

Results of cell cycle analysis were expressed as percentage of total examined cells and statistical analysis was performed by  $\chi^2$  test. Other results were expressed as  $\bar{x} \pm s$ . Differences between means were analyzed with Student *t* test for paired samples. A value of  $P < 0.05$  was considered statistically significant.

## RESULTS

### DNA fragmentation assay

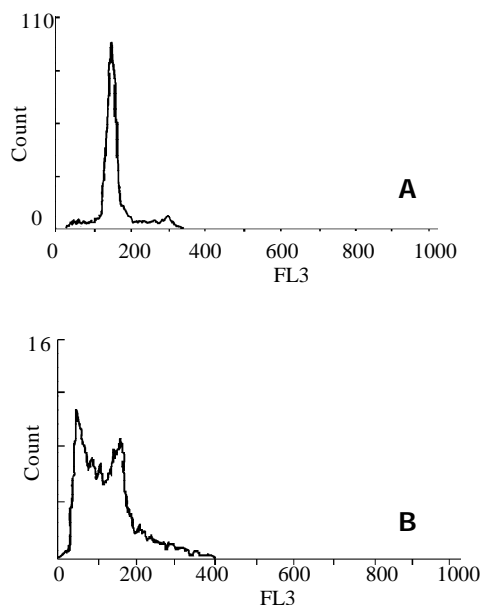
We investigated the role of FAK in the survival of HSC to rescue cells from apoptosis. An antibody to FAK that could inhibit its activation was used to test this hypothesis. This antibody binds to the COOH-terminal region of FAK, which contains the targeting sequence that is required for efficient recruitment of FAK to the focal adhesion<sup>[22, 32, 33]</sup>. HSC was treated with 30 mg · L<sup>-1</sup> anti-FAK antibodies in DMEM without serum. Genomic DNA fragment analysis performed 48-72 h after treatment demonstrating an oligonucleosomal DNA ladder for the treated cells, and the cells after treatment for 24 h showed minimal DNA ladder whereas the control cells in DMEM without the antibodies displayed no DNA degradation (Figure 1).



**Figure 1** Oligonucleosomal genomic DNA fragmentation. Lane M: DNA marker of PBR322; Lane 1: Control HSCs; Lane 2, 3, 4: HSC treated with anti-FAK antibodies for 24, 48 or 72 h.

### Flow cytometry

A predominant sub-G<sub>1</sub> population (39.8 %) characteristic of apoptosis was observed in anti-FAK antibodies treated HSCs for 72 hours by propidium iodide staining and flow cytometric analysis, while the control cells only had a (5.2 %) sub-G<sub>1</sub> population (Figure 2). There was significant difference between these two groups (39.8 % vs 5.2 %,  $\chi^2=1716.4$ ,  $P<0.001$ ). And a significant sub-G<sub>1</sub> population (16.5 %) was also observed in anti-FAK antibodies treated HSCs for 48 hours, while the control cells only had a (3.1 %) sub-G<sub>1</sub> population (16.5 % vs 3.1 %,  $\chi^2= 507.8$ ,  $P<0.001$ ). However, there was no significant difference between the 24-hour treatment and controls (3.1 % vs 2.7 %,  $\chi^2=1.4$ ,  $P>0.05$ ).



**Figure 2** Flow cytometric analysis of HSCs. A: Control HSC; B: HSC treated with anti-FAK antibodies for 72 h showed the presence of a hypodiploid (sub-G<sub>1</sub>) fraction, indicating DNA degradation.

### Cellular caspase-3 activity

Anti-FAK antibodies -induced apoptosis was accompanied by a significant time-dependent increase of caspase-3 activity (Table 1).

**Table 1** Caspase-3 activation in HSCs by anti-FAK antibodies (n=6)

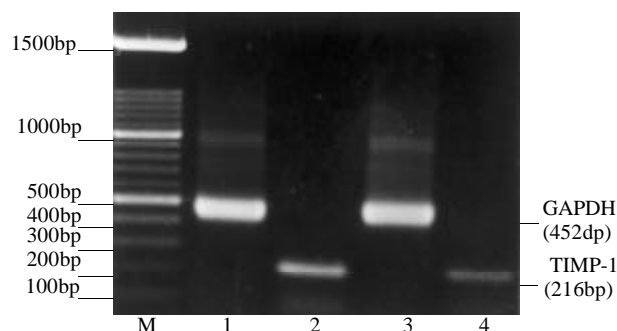
Groups	Caspase-3 activity (nmole·min <sup>-1</sup> ·g <sup>-1</sup> )
Control (24 h)	36.5±12.6
Treatment (24 h)	41.9±15.3
Control (48 h)	110.7±18.6
Treatment (48 h)	233.5±25.9 <sup>a</sup>
Control (72 h)	36.5±12.6
Treatment (72 h)	1208.5±76.4 <sup>a</sup>

<sup>a</sup> $P<0.05$ , vs control ( 233.5±25.9 vs 110.7±18.6,  $t=33.9$ ,  $P<0.05$ ; 1208.5±76.4 vs 36.5±12.6,  $t= 208.5$ ,  $P<0.05$ )

### Effect of anti-FAK antibody on the expression of TIMP-1 in HSCs

To evaluate whether the anti-FAK antibody affects the expression of TIMP-1 in HSCs, RT-PCR analysis was performed to detect the gene expression level of TIMP-1 after the treatment with anti-FAK antibodies in HSC. The mRNA

expression levels of cells that treated with the antibodies for 72 h was remarkably decreased as against that of the controls (0.07±0.01 vs 0.38±0.03,  $P<0.05$ , Figure 3). But there was no significant difference between the cells treated with the antibodies for 48 h or 24 h and their controls ( $P>0.05$ ).



**Figure 3** Electrophoresis analysis of RT-PCR product. Lane M: 100bp DNA ladder; Lane 1, 2: amplified from cDNA from the control HSCs; Lane 3, 4: amplified from cDNA from HSCs treated with anti-FAK antibodies for 72 h. The positive bands of 452bp and 216bp represented GAPDH and TIMP-1 respectively. Lane 4 showed decreased expression of TIMP-1 in HSC compared with lane 2.

### DISCUSSION

Liver fibrosis is characterized by an accumulation of extracellular matrix protein that impairs normal function with severity. It represents the common end point of the majority of chronic liver injuries. Ultimately, it results in distortion of the liver architecture (cirrhosis) which is associated with disturbance of liver function and significant morbidity and mortality<sup>[34 - 37]</sup>. At the cellular level there is now a wealth of evidence indicating that HSC represents the pivot of the fibrotic process. In the injured liver and during culture, quiescent HSCs transform from a retonoid rich pericyte-like cell to a myofibroblast-like cells (MFB). This so-called “activation” is associated with a loss of vitamin A droplets, increased proliferation, and sensitivity towards endothelins, increased production of ECM proteins, in addition to multiple alterations in gene expression<sup>[11-15]</sup>. Activation and transformation of HSCs into MFB may be viewed as a “wound-healing response”, however, little is known about the termination of this process, while HSC is abundant in the diseased liver tissue during fibrogenesis, and resolution of liver fibrogenesis is associated with reduced number of HSC. In the recovery from liver injury, apoptotic HSC was detected in parallel to a reduction in the total number of HSC within the liver tissue and an essential element of this recovery process is apoptosis of activated HSC<sup>[17-19]</sup>. Apoptosis (or programmed cell death) is the controlled mechanism by which cells are eliminated from tissue without eliciting an inflammatory response. Apoptosis of HSC may therefore play a central role in the resolution of fibrosis by eliminating the source of both the neomatrix and the metalloproteinase (collagenase) inhibitors and thereby facilitating net matrix degradation. Therefore, promoting HSC apoptosis may be a viable method to facilitate matrix degradation in fibrotic liver, thereby manipulating the fibrotic process. An understanding of the control of HSC apoptosis is important precisely because regulating this process may provide a novel therapeutic approach to the treatment of advanced hepatic fibrosis<sup>[18 - 20]</sup>.

Anchorage of cells to the ECM is mediated by integrins<sup>[38]</sup>, which not only mediated cell adhesion but also initiate

intracellular signal transduction<sup>[39]</sup>. A family of nonreceptor tyrosine kinases, composed of FAK, proline-rich tyrosine kinase (PYK-2), and integrin-linked kinase complex with the intracellular domain of integrins, leading to activation of various signaling pathways subsequent to integrin stimulation. Since the initial discovery and characterization of FAK, a number of different functions have been proposed for this unusual tyrosine kinase. FAK plays a pivotal role in transducing survival signals mediated by engagement of integrins with the ECM, enabling the cell to enter the cell cycle, thereby preventing apoptosis<sup>[1,40]</sup>. In rat HSCs, a soluble RGD peptide that blocks attachment to fibronectin and vitronectin triggered apoptosis in the serum-free condition, suggesting that integrin-mediated events can regulate death decisions in HSC<sup>[27]</sup>. Furthermore, the authors<sup>[27]</sup> reported that RGD peptides reduce the phosphorylation of FAK in HSC. Therefore in the current study we have described the role of FAK in the apoptosis in HSC.

Recently, a potential role for FAK in the suppression of apoptosis has been suggested in different cell types<sup>[7,41]</sup>. This study showed that anti-FAK antibodies induced apoptosis of culture-activated rat HSCs. This phenomenon displayed the classical features of apoptotic cell death (DNA fragmentation, cell cycle analysis), and accompanied by a significant increase of caspase-3 activity. Caspases, a family of the interleukin-1 $\beta$  converting enzyme (ICE) family of cysteine proteases, are key intracellular mediators of apoptosis<sup>[42, 43]</sup>. Caspase-3, also known as CPP32, Yama or apopain, is one of the principle caspases found in apoptotic cells<sup>[44]</sup>. Our observations suggest that induction of apoptosis in HSCs by anti-FAK antibodies may through the caspase-3 activation.

The data presented in this paper demonstrate that treatment of HSC with anti-FAK antibodies decreased the expression of TIMP-1 mRNA. Matrix metalloproteinases (MMP) and their specific inhibitors (TIMP) are thought to play an essential role in liver injury associated with tissue remodeling<sup>[45-47]</sup>, and it was reported that antisense oligonucleotides directed to TIMP-1 had some anti-hepatic fibrosis effect in the experimental immune hepatic rat models<sup>[48]</sup>. MMP or their inhibitors (TIMP) have been suggested to regulate apoptosis, and TIMP-1 inhibits cell death induced by hydrogen peroxide, adriamycin, or X-ray radiation<sup>[49]</sup>. It has recently shown that recovery from established experimental fibrosis can occur through the apoptosis of HSCs and is associated with reductions in liver collagen and expression of the TIMP-1 and TIMP-2<sup>[47]</sup>. Furthermore, FAK signaling pathway plays a pivotal role in the secretion of MMPs<sup>[50]</sup>. Therefore, the anti-FAK antibody regulated apoptotic pathway in HSCs might be triggered by interference with the TIMP-1 function.

In conclusion, integrin-ECM interactions influence apoptosis in rat HSC, and FAK is required for transducing survival signals from ECM in HSC. Our experiment provided a link between FAK and caspase-3, the expression of TIMP-1 and HSC survival. Thus the regulation of apoptosis may be very important in HSC biology.

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